#16

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	:	Group Art Unit: 1804
Skoultchi, A.	:	Examiner: Ziska, S.
Serial No.: 08/102,390	:	
Filed: August 5, 1993	:	
For: PRODUCTION OF PROTEINS USING HOMOLOGOUS RECOMBINATION	:	Attorney Docket No. 7639-017/CELL 3.2

DECLARATION OF MICHAEL LISKAY
UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

S I R:

I, MICHAEL LISKAY, do declare that:

1. I am a molecular biologist, specializing in the field of DNA recombination and repair. I received my Ph.D. in Genetics from the University of Washington in 1974. I currently hold the position of Full Professor in the Department of Molecular and Medical Genetics at the Oregon Health Sciences University in Portland, Oregon, where I carry out research in the field of molecular biology and genetics. One of the primary areas of my research interests during the

past 12 years relates to the investigation of homologous recombination events in mammalian cells. I have extensive experience in the study of homologous recombination and have published over 20 research papers on the topic. My curriculum vitae is attached hereto as Exhibit A.

2. Homologous recombination is a process in which two similar DNA sequences interact and undergo an exchange of genetic information. Different types of homologous recombination events are known to occur in cells, and can be classified into three general categories: extrachromosomal recombination (occurring between two extrachromosomal sequences such as two plasmids); intrachromosomal recombination (occurring between two chromosomal sequences); and targeted recombination or "gene targeting" (occurring between one extrachromosomal and one chromosomal sequence). A particular cell type could be very efficient at performing one type of homologous recombination, e.g., plasmid/plasmid recombination, but relatively incompetent to carry out another type of homologous recombination, e.g., gene targeting.

3. I have read the following patent applications, each of which was filed by Dr. Arthur Skoultchi and is entitled "Production of Proteins Using Homologous Recombination.":

(a) Application Serial No. 432,069 filed November 6, 1989; and

(b) Application Serial No. 07/787,390 filed November 4, 1991.

I understand that Application Serial No. 07/787,390 was filed as a continuation-in-part of Serial No. 432,069. Hereinafter I will refer to application Serial No. 07/787,390 as the "'390 parent application" and to application Serial No. 432,069 as the "'069 grandparent application." I also understand that the above-captioned application is a continuation of the '390 parent application.

4. Dr. Skoultchi's invention, as described in both the '390 parent application and the '069 grandparent application, involves the targeted integration of a regulatory sequence, such as a promoter/enhancer, and/or an amplifiable gene into a mammalian host cell genome to activate and/or enhance expression of a target gene. To this end, a unique approach involving targeted homologous recombination is described for engineering the target genes in mammalian host cells so that the activated and/or amplified gene product is expressed. Where the engineered cell is a continuous cell line, it can be used for large-scale production of that gene product in cell culture. However, each patent application also explains that where the engineered mammalian cell is a primary cell that does not grow readily in culture, the entire activated gene (including the engineered expression and amplification elements) can be transferred to a secondary expression host cell that is more efficient for large scale production. ('390 parent application at p. 3, lines 11-25; p. 4, lines 5-30; p. 10, line 35 to p. 12, line 16; and '069 grandparent application at p. 3, lines 15 to p. 4, line 35; p. 10, line 18 to p. 11, line 31).

5. Each patent application describes the use of targeted homologous recombination to integrate a promoter (referred to as the "transcriptional initiation region") and/or enhancer different from the wild-type promoter/enhancer of the target gene into the mammalian host cell genome, so that the integrated promoter/enhancer, not the wild-type native promoter/enhancer, controls expression of the target gene. ('390 parent application at p. 7, lines 3-35; and '069 grandparent application at p. 6, line 2 to p. 7, line 21). Likewise, each application describes the integration of an amplifiable gene proximal to the target gene so that both the integrated amplifiable gene and the engineered target gene can be amplified to increase target gene expression. ('390 parent application at p. 4, lines 5-29; p. 5 lines 11-23; p. 11, line 32 to p. 12, line 16; and '069 grandparent application at p. 3, line 31 to p. 4, line 17; p. 4, line 36 to p. 5, line 10).

6. The invention described in Dr. Skoultchi's patent applications is illustrated by way of examples using DHFR as the amplifiable gene to enhance the expression of a target gene called tissue plasminogen activator or "t-PA" ('390 parent application at p. 12, line 20 to p. 14, line 36; and '069 grandparent application p. 12, line 1 to p. 14, line 26), and the CMV (cytomegalovirus) promoter/enhancer to control the expression of a target gene called erythropoietin or "EPO" ('390 parent application at p. 15, line 1 to p. 19, line 29).

7. The invention described in the '390 parent and '069 grandparent applications affords a clever and powerful approach to gene expression that I wish I had thought of myself. It provides several advantages over traditional recombinant DNA approaches to gene expression, e.g., where expression vectors or cassettes are used to direct the expression of cloned cDNAs. The invention can obviate the need to clone the entire gene itself, and can allow for expression of large genes that are difficult to clone, or impossible to clone in cloning and expression vectors that are commonly used to clone and express cDNAs. Moreover, the invention provides for the efficient expression of target genes by including a number of components absent from cDNA constructs, e.g., introns, upstream and downstream sequences -- all of which contribute to efficient expression of the gene product. Furthermore, the invention described provides for versatility and safety in the selection of target genes and mammalian hosts -- the practitioner is not confined to target genes that are present in transformed cell lines, but can target human genes in normal, untransformed human host cells. Moreover, difficulties or obstacles encountered in activating target genes in one cell type can be overcome by switching to a different cell type for targeting. Where the host cell is a primary cell that is not immortalized, production of the targeted gene product in continuous culture can be accomplished by the transfer of the activated gene to an expression host cell system used for commercial production.

8. I have been asked to consider whether the teachings of the '069 grandparent application are confined to the use of enhancers as the regulatory sequence that is integrated into a host cell genome to activate expression of a target gene. In reaching my conclusions, I considered how a molecular biologist would have understood and interpreted the teachings of the '069 grandparent application in 1989, the year the application was filed. Based upon my review of the '069 grandparent application, and taking into account the knowledge and understanding of one skilled in the art of molecular biology in 1989, I have concluded that the teachings of the '069 grandparent application are <u>not</u> limited to the targeted integration of enhancers, but include the targeted integration of any and all promoter elements different from the wild-type or native promoter that control expression of the target gene.

9. At the outset, the written description of the "regulatory sequences" that can be targeted into host cells to activate target gene expression is identical in both of Dr. Skoultchi's patent applications. (See '069 grandparent application at p. 3, lines 15-21 and p. 6, line 25 to p. 7, line 21; and '390 parent application at p. 3, lines 12-17 and p. 7, lines 1-35). This description is not confined to the use of enhancers, but includes the targeted integration of promoter sequences, referred to in the application as "transcriptional initiation regions" different from the wild-type promoter sequence that normally controls expression of the target gene. The reference to promoters (or promoter

sequences) as "transcriptional initiation regions" is entirely consistent with the then commonly accepted definition of a promoter; i.e., the region(s) of DNA necessary for regulated and efficient initiation of transcription. One skilled in the art of molecular biology in 1989 would certainly have understood the term "transcriptional initiation region" used in Dr. Skoultchi's patent applications to mean promoter (or promoter sequences), and would not have interpreted the teachings of the '069 grandparent application to be confined to the targeted integration of enhancers.

10. I have reviewed the following publications which, I understand, have been considered by the Examiner of Dr. Skoultchi's patent application:

(a) Primrose, "Principles of Gene Manipulation," 2d ed., Univ. Calif. Press, Berkeley Los Angeles 1981 ("Primrose");

(b) Watson et al., Recombinant DNA A Short Course, W.H. Freeman & Co., NY, 1983, pp. 41 and 47 ("Watson"); and

(c) Lewin, Genes, pp. 188-194 ("Lewin").

11. The descriptions and definitions of promoters found in Primrose, Watson and Lewin are consistent with my understanding of the teachings of Dr. Skoultchi's application. In particular, Primrose defines a promoter as "a region of a DNA molecule at which RNA polymerase binds and initiates transcription." Watson and Lewin are consistent with this interpretation. Although Watson and Lewin describe the

"dissection" of a promoter into its component parts, e.g., the RNA polymerase recognition site, the RNA polymerase binding site and the transcriptional start site, these references also reflect that the transcriptional initiation region as a whole represents the promoter.

12. I have reviewed the following publications which, I understand, have been considered by the Examiner of Dr. Skoultchi's patent application:

(a) Thomas & Capecchi, 1987, Cell 51:503-512 ("Thomas");

(b) Thompson et al., 1989, Cell 56:313-321 ("Thompson");

(c) Song et al., 1987, Proc. Natl. Acad. Sci. USA 84:6820-6824 ("Song");

(d) Liskay et al., 1984, CSHSQB 49:183-189 ("Liskay");

(e) Anderson, 1984, Science 226:401-409 ("Anderson").

13. Thomas describes the use of targeted homologous recombination to disrupt and inactivate a target gene in mouse embryonic stem (ES) cells. In particular, a DNA construct containing a bacterial neo^r gene controlled by the thymidine kinase (TK) promoter was prepared in vitro using standard recombinant DNA techniques. This construct was used to disrupt expression of the endogenous Hprt gene in the mouse ES cells via homologous recombination-mediated insertion of the neo^r gene.

14. Thompson describes the correction of a defect in the Hprt gene in mutant mouse ES cells. The correct Hprt sequence contained in a plasmid was introduced into mutant ES cells containing a deletion mutation in the coding region of Hprt. The deletion mutation in the Hprt gene was corrected.

15. Song describes homologous recombination events that occur between an artificial defective target gene previously inserted into the host cell chromosome via random integration, and a homologous sequence contained in a plasmid introduced into the cell. In particular, a defective bacterial gene, neo^r, was integrated, via random events, into the host cell genome. Transfection of the cells with a plasmid DNA containing a neo^r gene carrying a different mutation resulted in "targeted" correction of the defective neo^r gene located in the host cell chromosome.

16. Liskay, one of my own publications, relates to a study of intrachromosomal homologous recombination i.e., recombination events between closely linked duplicated genes located in a chromosome -- the study reported in my publication did not relate to targeted homologous recombination. In particular, a plasmid DNA molecule containing two different mutant copies of a selectable gene was integrated, via random events into the genome. This produced a host cell genome that contained one copy of the recombination substrate, i.e., a gene duplication. These cells were used to measure frequencies of both non-reciprocal

and reciprocal intrachromosomal recombination in cell lines containing a single direct gene repeat.

17. Anderson is a review article relating to prospects for human gene therapy, and describes different procedures for delivering and inserting a normal gene into an organism to correct a genetic defect; i.e., gene replacement therapy. The design of recombinant DNA vectors, such as viral or plasmid DNAs, containing various promoters and/or enhancers to control expression of the cloned gene is described, as are various systems for delivering the cloned genes to cells; e.g., viral systems, chemical-mediated uptake, fusion (DNA-loaded liposomes), and physical methods (microinjection or electroporation).

18. I have been asked to consider whether the foregoing references, considered individually or in combination, suggest the invention described and claimed in Dr. Skoultchi's application. Based upon my review of the references, and taking into account the knowledge that one skilled in the art of molecular biology would have had in 1989, I have come to the conclusion that the references cited above do not suggest Dr. Skoultchi's invention. My reasons are detailed below.

19. None of the foregoing references suggest that targeted homologous recombination should or could be used for gene activation. For example, the references do not suggest the targeted recombination of a regulatory sequence such as a

promoter and/or enhancer, different from the native promoter or enhancer, into a mammalian host cell genome so that expression of the target gene is controlled by the integrated regulatory sequence. Likewise, the references do not suggest the targeted recombination of an amplifiable gene into a mammalian host cell genome proximal to the target gene so that the target gene is amplifiable. While it is true that as of 1989 homologous recombination was known, that promoters/enhancers were known and used to control expression of cloned genes, and that amplifiable genes were known, the above-identified references do not suggest to one skilled in the art of molecular biology the unique combination of the elements or features of Dr. Skoultchi's invention as taught in his patent applications to control the expression of a gene via targeted gene activation.

20. Thomas describes experiments in which targeted homologous recombination was used to disrupt, inactivate or "knock-out" the expression of a selectable gene, Hprt, in mouse ES cells. This is quite the opposite of Dr. Skoultchi's invention which utilizes homologous recombination to activate or enhance target gene expression.

21. Thompson and Song describe experiments in which targeted homologous recombination was used to correct a mutant gene resident in the host cell chromosome -- Thompson corrected mutations in the Hprt structural gene in mouse ES cells, whereas Song corrected mutations in an artificial target, i.e., a defective bacterial gene, which had been

randomly integrated into the mammalian host cell genome. Neither suggests the use of targeted homologous recombination to activate target gene expression.

22. Although Thompson alludes to the future possibility of manipulating the expression of genes in animals by targeting unspecified changes to their control sequences, (Thompson, p. 313, "Introduction", lines 3-9), he does not suggest the wholesale replacement of the control sequence of the target gene with an exogenous control sequence different from the wild-type or native sequence to activate target gene expression. When read in context with the entire article, it appears that Thompson's changes to the control sequences refers to targeting mutations to the Hprt promoter in order to "map" the promoter, i.e., to determine the mechanism by which expression of the Hprt gene is elevated in brain tissue. (Thompson, p. 319, column 2, lines 55-58). Finally, Thompson does not suggest that targeted homologous recombination could be used to activate gene expression for purposes such as large scale protein production in mammalian cell culture, and confines his projections to manipulating mutations to genes in ES cells. (Thompson, p. 320, column 1, lines 24-26).

23. Liskay does not relate to the use of targeted homologous recombination at all, much less its use for gene activation. The purpose of our study, as explained in the Liskay reference itself, was to investigate the frequencies of intrachromosomal homologous recombination events between closely linked duplicated genes lying within a chromosome.

This was accomplished by engineering a cell line containing a mutant pair of genes integrated into the host cell genome via random events. The ability of a host cell to carry out intrachromosomal homologous recombination did not necessarily suggest anything regarding the capacity of that cell to carry out gene targeting.

24. Anderson, which relates to gene therapy using standard recombinant DNA in vitro approaches for delivering exogenous cloned genes into host cells, does not suggest the use of targeted homologous recombination to activate target gene expression. In fact, Anderson teaches away from the use of homologous recombination in mammalian cells, characterizing it as a "formidable task" (Anderson, p. 404, column 2, first full paragraph, lines 28-42).

25. I have been asked to consider whether, in view of the teachings of the above-identified references, one of ordinary skill in the art of molecular biology in 1989 would have had a reasonable expectation of successfully carrying out targeted gene activation in mammalian cells. Based upon the teachings, experiments and data described in the foregoing references, and taking into account the knowledge that one of ordinary skill in the art of molecular biology would have had in 1989, I have come to the conclusion that the references would not have provided such a person with a reasonable expectation of successfully activating gene expression using targeted homologous recombination in mammalian cells.

26. In 1989, the different types of homologous recombination known to occur in cultured mammalian cells were extrachromosomal (plasmid/plasmid), intrachromosomal, and targeted homologous recombination. It was also known that the frequency and efficiency of each type of homologous recombination varied for any given cell, and between cells. For example, it was understood that a cell could be competent to perform one type of homologous recombination, such as extrachromosomal or intrachromosomal recombination, but incompetent to carry out gene targeting. The competence of any given cell for a particular type of homologous recombination was unpredictable. At that time, targeted homologous recombination was known to occur efficiently in lower organisms such as bacteria and yeast. However, targeted homologous recombination was, in general, viewed as a rare event and difficult task in most commonly used mammalian cells. Given this understanding at the time, there was no motivation to use targeted homologous recombination to engineer target genes in mammalian cells as taught by Dr. Skoultchi. This view is confirmed by the Anderson reference which teaches away from the use of homologous recombination in mammalian cells, characterizing it as a "formidable task".

27. Both Thomas and Thompson reported studies involving targeted homologous recombination in mouse ES cells to either disrupt or knock-out target gene expression (Thomas) or to correct a mutation in a structural gene (Thompson). The prevailing view held at the time was that ES cells were somehow special, in that these embryonic cells might possess

unique capabilities to perform targeted homologous recombination more efficiently than other mammalian cells. In this regard, the ES cell system was viewed as more similar to bacterial and yeast systems which were thought to be efficient at targeted homologous recombination. Results obtained in the mouse ES system were not generally extended, extrapolated or considered predictive of results that could be achieved in other mammalian cells. Thus, based upon the results reported by Thomas and Thompson in ES cells, one of ordinary skill in the art would not have had a reasonable expectation of successfully using targeted homologous recombination in other mammalian cells to activate gene expression.

28. Song describes studies of homologous recombination events involving artificial targets; i.e., exogenous genes integrated into the host cell genome via random events. Song reported the use of targeted homologous recombination to correct a defective artificial target gene. However, success or failure of targeted recombination between such artificial resident defective genes and their corrected counterparts borne on plasmids was not considered necessarily predictive of success or failure for recombination events involving endogenous native genes. Indeed, attempts to target mutations into endogenous native genes were not as successful as the results achieved for Song's artificial targets. I know of only one report as of 1989 relating to gene targeting for modifying or correcting endogenous genes in mammalian cells, and the results achieved were modest: Smithies et al., 1985, Nature 317:230-234 ("Smithies"). Smithies indicates that the

frequency for targeting native endogenous genes is much lower than that for artificial target genes, and indicates that results may depend upon the location of the target gene and the host cell, e.g., whether the target gene was located within an accessible chromosomal locus. (See, for example, Smithies at p. 232 "Preliminary Work"). Therefore, the experiments involving artificial targets reported in Song would not have provided one or ordinary skill in the art with a reasonable expectation of successfully targeting native endogenous genes in mammalian host cells, much less for purposes of gene activation.

29. Liskay, like Song, describes studies of homologous recombination events involving artificial targets. However, Liskay is even less relevant to Dr. Skoultchi's invention since it involves intrachromosomal, not targeted, homologous recombination. Results achieved and frequencies reported for intrachromosomal events were not necessarily considered predictive of success for gene targeting.

30. In conclusion, on the basis of all the foregoing considerations, it is my opinion that Dr. Skoultchi's invention represents, for its time, a unique and unusual approach to gene expression. Although each of the tools utilized in the invention were known individually, e.g, gene targeting, promoters, enhancers and amplifiable genes, the unique combination of the various elements involved had not been suggested; i.e., the use of gene targeting to engineer exogenous promoters/enhancers and/or amplifiable

genes into mammalian host cells so that expression of endogenous target gene is controlled, activated or enhanced by the exogenous integrated sequence. None of the references above suggested the use of targeted recombination for combined gene activation and amplification. Moreover, the experiments and data reported in the references would not have provided one of ordinary skill in the art of molecular biology with a reasonable expectation that targeted gene activation would work in mammalian cells. Only with hindsight, after having learned of Dr. Skoultchi's unique and unusual combination of elements and approach to gene expression could his invention be said to be obvious.

31. I declare further that all statements made in this Declaration of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Feb 3 1995

Michael Liskay